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Molecular cloning and characterization of a cDNA encoding the prophenoloxidase gene in *Bombyx mandarina*

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Abstract: Phenoloxidase plays an important role in defense mechanisms in insect immunity. The complemental deoxyribonucleic acid (cDNA) of *Bombyx mandarina* prophenoloxidase (PPO) gene was cloned by means of reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE). The cDNA was 2 134 bp in length and contained an open reading frame (ORF) of 2 082 bp which encoded 693 amino acid residues. The deduced amino acid sequence showed a high identity to the reported sequence of PPO2 from other Lepidopterous insects and shared the typical structural features of PPO from other insects. The results of RT-PCR showed that PPO mRNA was expressed in the integument, haemocyte, head, testis, ovary, fat body and midgut of 5th instar larvae and at different developmental stages of *B. mandarina*. These results provide molecular basis for further studying the function of ppo gene in *B. mandarina*.

Key words: *Bombyx mandarina*; prophenoloxidase gene; cloning; sequence analysis; tissue expression

1 INTRODUCTION

Phenol oxidase is a copper protein that catalyzes the oxygenation of monophenols to O-diphenols and the oxidation of O-diphenols to the corresponding O-quinones. The enzyme is present in both animals and plants as a key enzyme for melanin synthesis (Mason, 1955, 1965). In insects, this enzyme was found in hemolymph and cuticle as inactive precursor (prophenol oxidase, pro-PO), which was activated through a limited proteolysis by a specific serine-type proteinase that in itself is a zymogen form (Lai-Fook, 1966; Ashida, 1971; Ashida and Brey, 1995). In such a mode for enzyme activity regulation, the organism not only resists invasion by microbes effectively but also avoids the harm of excessive activation under the normal physiological conditions. Insect PO is implicated in defense reaction, such as wound healing and melanization during pathogen or parasite invasion (Andersen *et al*., 1996). For this reason POs have been characterized, and their encoding genes have been cloned in insect species such as *Manduca sexta*, *Drosophila melanogaster*, *Bombyx mori*, *Anopheles gambiae* and *Aedes aegypti* (Fujimoto *et al*., 1995; Kawabata *et al*., 1995; Jiang *et al*., 1997; Müller *et al*., 1999; Taft *et al*., 2001). However, so far the prophenoloxidase gene has not been reported yet from *B. mandarina*. We report here the molecular cloning, the sequencing of the overall open reading frame (ORF) region and the evaluation of the specific expression pattern of prophenoloxidase in *B. mandarina*.

2 MATERIALS AND METHODS

2.1 Experimental animal

The *B. mandarina* obtained from the Beibei Silkworm Breeding Farm, Chongqing, were used for all experiments.

2.2 RNA extraction and cDNA synthesis of the first chain

The materials were sampled and freeze-dried before RNA extraction. Total RNA was extracted from tissues of *B. mandarina* larvae (5th instar, day 3) and from the whole *B. mandarina* body at different developmental stages using TRIzol (Invitrogen, USA) according to the manufacturer's instructions. cDNAs corresponding to the first strand were reversely transcribed from the total RNA using M-MLV reverse transcriptase (Promega, USA). The sample used were

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as follows: midgut, head, ovary, testis, silk gland, hemocytes, fat body and integument from 5th instar larvae of Day 3, whole body of newly hatched larva, 1st instar larvae, 2nd instar larvae, 3rd instar larvae, 4th instar larvae, 5th instar larvae of Day 1 – 6, prepupa and pupae.

2.3 Molecular cloning of the prophenoloxidase gene in *B. mandarina* and DNA sequencing

The forward and reverse degenerate primers (5′-GCGTA(CT)TGGCGAGA(AG) GA(TC)AT- 3′and 5′-GAAAAC(AG)TC(AG)TC(TGA)AT(GA)TAGG-3′), based on the conserved amino acid sequence of prophenoloxidase gene reported from Lepidopterous insects, were designed to amplify the conserved copper binding region in *B. mandarina*. Cycling conditions were denaturation at 94℃ for 5 min, followed by 30 cycles of 94℃ for 0.5 min, annealing at 55℃ for 0.5 min and extension at 72℃ for 1 min, with a final 10 min extension at 72℃. The PCR products from midgut were separated on 1% agarose gel, purified with Gel Extraction Mini Kit (Watson, China), ligated into the pMD18-T vector (TaKaRa BioTech, China), and transformed into competent cells of *E. coli* strain DH5α according to the manufacture's introduction. Positive clones were sequenced on ABI-3100 DNA sequencer.

2.4 The rapid amplification of cDNA ends reaction

The 5′-GSP and 3′-GSP (Gene specific primer, GSP) (5′ -CCGAGTCTGCCCGTTTGGCAATCT AAC -3′ and 5′-CGTCGTGGGGAGTTGTTCTTCTATATGC-3′), based on the obtained middle part of the cDNA of prophenoloxidase gene from *B. mandarina* in this paper, were designed to amplify the overall ORF region. Cycling conditions were denaturation at 94℃ for 3 min, followed by 5 cycles of 94℃ for 5 s and extension at 72℃ for 3 min, 5 cycles of 94℃ for 5 s, annealing at 70℃ for 10 s and extension at 72℃ for 3 min, 25 cycles of 94℃ for 5 s, annealing at 68℃ for 10 s and extension at 72℃ for 3 min, with a final 10 min extension at 72℃. After the rapid amplification of cDNA, molecular cloning was performed according to the manufacture's introduction (SMART RACE cDNA amplification kit, Clontech, China) and the protocols described in the preceding section.

2.5 Analysis of nucleotide and amino acid sequences

All the cDNAs were assembled into a consensus sequence containing the complete open reading frame (ORF) with PHRAP software (http://www.phrap.org). The overall ORF as well as translation of the cDNA nucleotide sequence to a protein sequence was performed by translation tool from ExPaSy (http://www.expasy.org/tools/dna.html). Sequences of the proteins from *B. mandarina* and other insect species were aligned using Clustal X (Thompson *et al.*, 1997), and functional domains were predicted in SMART web site (http://smart.embl-heidelberg.de/).

2.6 Analysis of expression pattern by RT-PCR

RT-PCR was performed with the forward and reverse primers (5′-GCGTACTGGCGAGAAG ATAT-3′ and 5′-GAAAACGTCATCGATGTAGG-3′), as mentioned above for testing the expression pattern of the ppo gene in *B. mandarina*. Program procedure was: 1 cycle of 94℃ for 5 min, 25 cycles of 94℃ for 0.5 min, 55℃ for 0.5 min, and 72℃ for 1 min, and a final extension of 10 min at 72℃. Silkworm actin (A3) mRNA was chosen as the internal control. PCR products were analyzed on 1% agarose gel.

3 RESULTS

3.1 Molecular cloning and nucleotide sequence analysis of the prophenoloxidase gene in *B. mandarina*

The RT-PCR and RACE have been used to clone the gene, with cDNA from larva midgut as template. The band appeared on the agarose gel with a length of approximately 639 bp, designated as fragment of the conserved copper binding region of *B. mandarina* prophenoloxidase gene (Fig. 1)

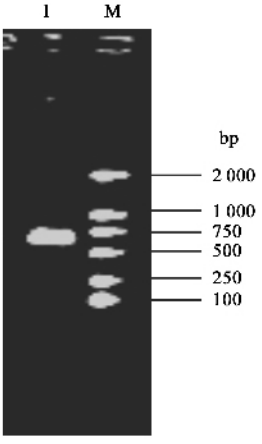

Fig. 1 Agarose gel analysis of *Bombyx mandarina* ppo partial cDNA fragments amplified by RT-PCR.
A band obtained after PCR amplification (lane 1).
M represents the DL2000 marker.

After the band was cloned into pMD18-T vector and thoroughly sequenced, RT-PCR and RACE were performed. The sequence of the conserved copper binding region, the cDNA and the complete open reading frame (ORF) were obtained with a length of 639, 2 134 and 2 082 bp, respectively (GenBank accession number: EU047703). The intact ORF, lies in 422 082 nt, encodes 693 amino acid residues,

initiates at the ATG initiation codon and terminates at the TAA termination codon. Functional domain prediction showed that it contains five possible N-linked glycosylation sites, residues 26 – 28, 64 – 66, 462 – 464, 494 – 496, 680 – 682, indicated in Fig. 2 by curve, and two putative highly conservative copper-binding regions A and B, which correspond to residues 101 – 245 and 346 – 413, underlined in Fig. 2 by horizontal lines, including six conserved boxed histidine residues at 213, 217, 243, 366, 370, and 406 position, respectively. In addition, a possible cleavage site for proteolytic activation of the enzyme, between residues 51 (arginine) and 52 (phenylalanine) of the deduced sequence, was indicated in Fig. 2 by an arrow. The sequence motif around the peptide bond to be hydrolyzed was Asn-Arg-Phe-Gly. The cDNAs of *B. mandarina* prophenoloxidase gene lacked signal peptide sequences. Alignments of the cDNAs with the genome sequence showed *B. mandarina* prophenoloxidase gene in this study is present at a single copy in the silkworm genome, suggesting that the primary transcript of this gene generates only one mature mRNA isoform. Further alignment with genome sequence demonstrated that *B. mandarina* prophenoloxidase gene comprises eleven exons and ten introns, and the exon-intron junction matches the classical GU-AG rule. Interestingly, it is notable that the structure of this gene is highly conserved in Lepidopterous insects from *B. mori* to *Helicoverpa armigera*.

3.2 The alignment of amino acid sequences of PPO gene family between *B. mandarina* and other insects

The alignment of amino acid sequences showed different degree of similarity among members of the ppo gene family. The amino acid sequence of *B. mandarina* ppo has higher homologies with the amino acid sequences of ppo2 than that of ppo1 of other Lepidopterous insects. For example, *B. mandarina* ppo has 76%, 49% identity and 87%, 69% similarity to *M. sexta* ppo2 and ppo1 gene, respectively (AAC37243 and AAC05796). *B. mandarina* ppo has 76%, 48% identity and 87%, 67% similarity to *Galleria mellonella* ppo2 and ppo1 gene, respectively (AAQ75026 and AAK64363). *B. mandarina* ppo has 73%, 49% identity and 85%, 66% similarity to *Hyphantria cunea* ppo2 and ppo1 gene, respectively (AAC34256 and AAC34251). *B. mandarina* ppo has 99%, 50% identity and 99%, 69% similarity to *B. mori* ppo2 and ppo1gene, respectively (BAA08369 and BAA08368). *B. mandarina* ppo has 79% identity and 88% similarity to *H. armigera* ppo2 gene (AAZ52554). Also, the amino acid sequences of *B. mandarina* ppo has high homologies with that of ppo from Dipterous insects. For example, *B. mandarina* ppo has 52% identity and 70% similarity to *Musca domestica* ppo gene (AAR84669). *B. mandarina* ppo has 52% identity and 69% similarity to *D. melanogaster* ppo gene (AAF59001). *B. mandarina* ppo has 42% identity and 60% similarity to *A. gambiae* ppo gene (EAA44337). Furthermore, *B. mandarina* ppo shares the same domain architecture as that of arthropod hemocyanins: domains highly homologous to the copper-binding site sequences (copper-binding sites A and B). Phenol oxidases from prokaryotes, fungi, and vertebrates have sequences homologous to only the copper-binding site B of arthropod hemocyanins (Kawabata *et al*., 1995).

3.3 Tissue distribution and developmental expression of *B. mandarina* ppo

To investigate the expression profile of *B. mandarina* ppo, RT-PCR using cDNA from different tissues and developmental stages from newly hatched larva to pupae (see materials and methods) as template was performed. Results showed *B. mandarina* ppo could be detected in most of the tissues and all developmental stages, while it could not be detected only from silk gland. But expression of *B. mandarina* ppo gene was much higher in head, hemocytes, midgut, and testis than in ovary and integument, much lower in fat body (Fig. 3).

It was very interesting to find that *B. mandarina* ppo gene was widely expressed in all the tested developmental stages. The gene expression profiles analysis of mRNA isolated from whole body at different developmental stages revealed that the transcripts of *B. mandarina* ppo gene was detected during all stages, and the abundance was almost the same throughout the tested developmental stages.

4 DISCUSSION

Using a RT-PCR and RACE-based cloning strategy, we isolated the mRNA, and obtained the full open reading frame sequence from a single *B. mandarina* ppo gene. There are two ppo genes (ppo1 and ppo2) or more, two highly conservative copper-binding regions A and B, no signal peptide sequences in most reported insects, including *M. sexta*, *D. melanogaster*, *B. mori*, *A. gambiae*, and *Aedes aegypti etc* (Fujimoto *et al*., 1995; Kawabata *et al*., 1995; Jiang *et al*., 1997; Müller *et al*., 1999; Taft *et al*., 2001). Similar results have been obtained in this study. Homology analysis showed higher similarity of the sequences of amino acid residues deduced in this paper for *B. mandarina* ppo gene (85% – 99%) with those of ppo2 from other Lepidopterous insects, of

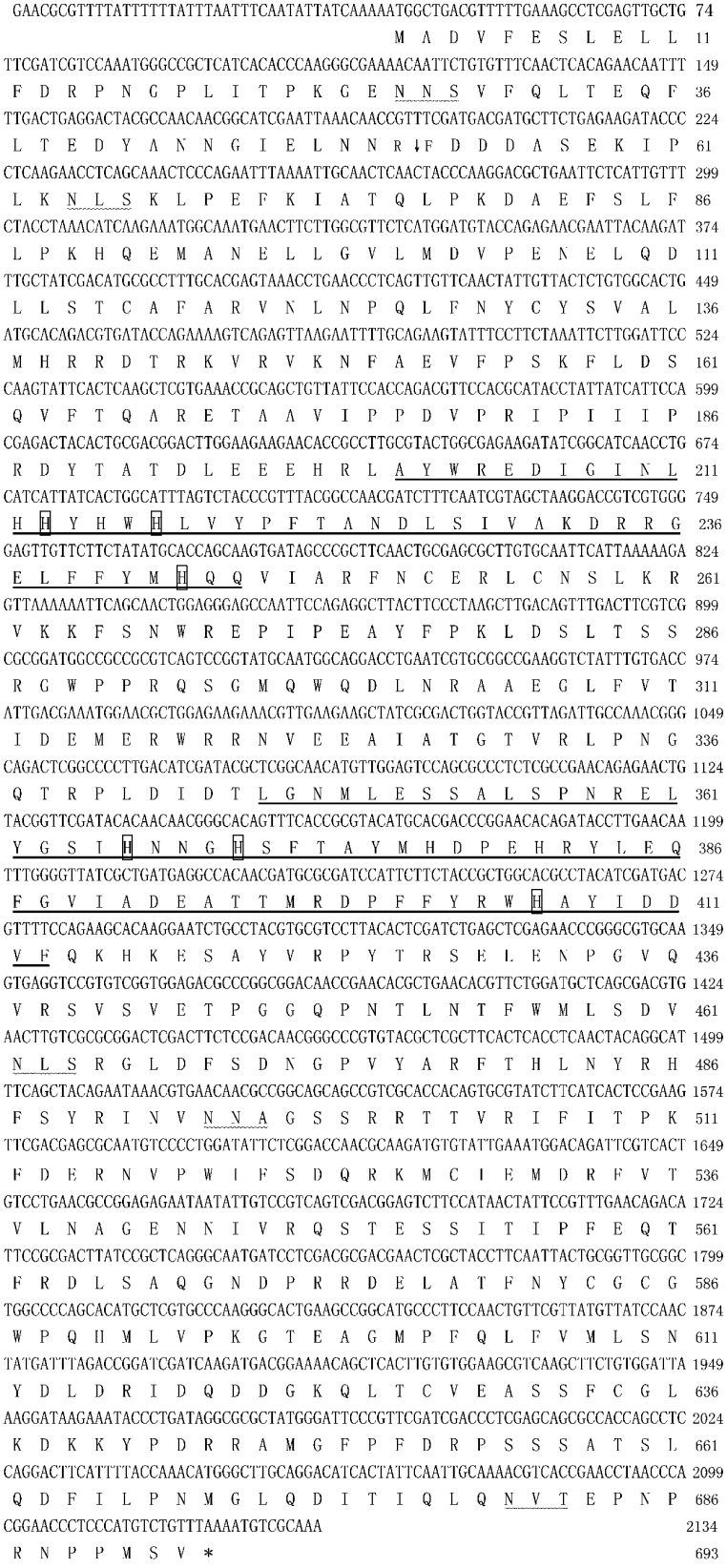

Fig. 2 Nucleotide and deduced amino acid sequence of *Bombyx mandarina* prophenoloxidase gene (GenBank accession number: EU047703).

The amino acid sequence is given below the nucleic acid sequence. Numbers on the right refer to the last amino acid residue and nucleotide, respectively, on each line. Termination codon is indicated by asterism. An arrow indicates a possible cleavage site for proteolytic activation of the enzyme. Possible N-linked glycosylation sites are indicated by curve. Two putative copper-binding sites are underlined. Six conserved histidine residues are boxed.

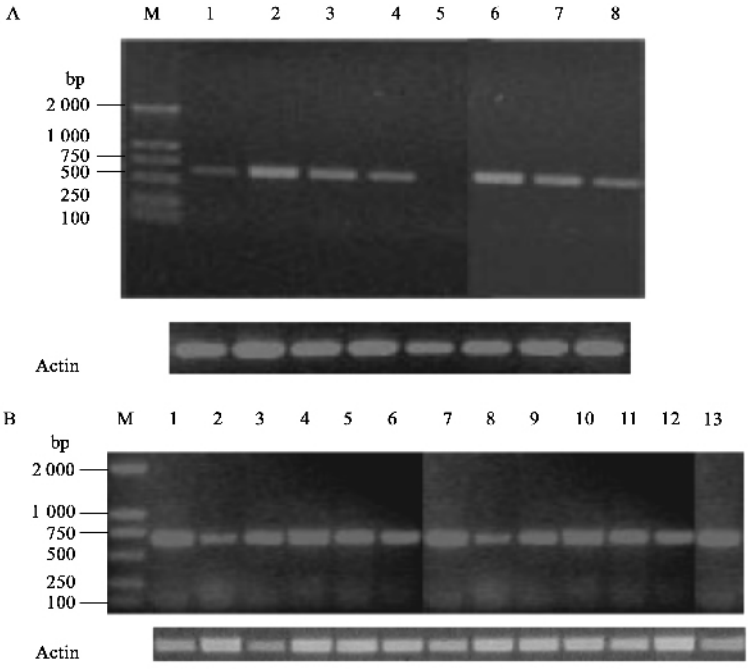

Fig. 3 Expression profile of *Bombyx mandarina* ppo in different tissues and at different developmental stages.
A. Expression of *B. mandarina* ppo in multiple tissues of 5th instar larvae. Fat body (lane 1), head (lane 2), testis (lane 3), ovary (lane 4), silk gland (lane 5), hemocytes (lane 6), midgut (lane 7) and integument (lane 8) were used. B. Expression of *B. mandarina* ppo in whole body of newly hatched larva (lane 1), 1st instar larvae (lane 2), 2nd instar larvae (lane 3), 3rd instar larvae (lane 4), 4th instar larvae (lane5), 5th instar larvae of Day 1 – 6 (lane 6 to lane 11), prepupa (lane 12), and pupae (lane 13) were detected. M represents the DL2000 marker.

which the highest similarity was found with the ppo2 sequences of *B. mori* (99%). Thus, the *B. mandarina* ppo gene addressed in the present study was probably the ppo2 gene. Future study will clarify whether there is ppo1 gene in *B. mandarina*. Furthermore, as mentioned above, this high homology between *B. mandarina* ppo and *B. mori* ppo2 again further supports the conclusion that *B. mori* has originated from *B. Mandarina*, and *B. mori* has evolved from *B. mandarina*.

RT-PCR analysis indicated that the expression pattern of *B. mandarina* ppo is similar to that of other insects. For example, the *A. gambiae* ppo mRNA expressed in the midgut and hemolymph (Müller *et al*., 1999). The *A. stephensi* ppo mRNA expressed in the larva, pupae, and adult stage. However, the expression was high in the hemocytes, integument, and fat body, but much lower in ovary, and no expression in midgut (Cui *et al*., 2000). The *Apis mellifera* ppo mRNA expressed in tissues like integument. (Anete *et al*., 2005). Whereas, the *Armigeres subalbatus* ppo mRNA expressed only in hemocytes, and no expression in midgut and ovary *etc*. (Cho *et al*., 1998). These results indicated although ppo was originally found in the hemolymph, it was not the only site for ppo expression. The expression profile of ppo from distinct tissue sites may be different among insect species. This implies that ppo from different tissues or different species may have different physiological functions. The present results support the above argument too.

Phenol oxidase (PO) is important in cuticular sclerotization and in defense against pathogens and parasites by insects (Jiang *et al*., 1998); however, cuticular sclerotization and defense reaction are essential to insect survival. Hence, molecular cloning and expression profile analysis of *B. mandarina* ppo gene provide molecular basis for functional protein studies, biological control, origin and evolution of *B. mori*, and so on. These studies have not only the theoretic signification but also the extensive practical prospect.

5 CONCLUSION

From the results of this investigation, it can be concluded that there is an open reading frame (ORF) of 2 082 bp which encoded 693 amino acid residues in *B. mandarina* prophenoloxidase (PPO) gene. The deduced amino acid sequence showed a high identity to the reported sequence of PPO2 from other Lepidopterous insects. The mRNA was expressed in the integument, haemocyte, head, testis, ovary, fat body and midgut of 5th instar larvae and at different developmental stages of *B. mandarina*. Further studies are required to confirm whether there is ppo1 gene in *B. mandarina* and clarify how its physiological functions are different from those of *B. mandarina* prophenoloxidase gene.

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野桑蚕酚氧化酶原基因 cDNA 的分子克隆及其特征

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摘要:酚氧化酶在昆虫的免疫防御机制中起着重要作用。利用 RT-PCR 和 RACE 方法,克隆了野桑蚕酚氧化酶原基因,获得了其 cDNA 序列。该序列长 2 134 bp,含有一个 2 082 bp 的完整开放阅读框,编码一个由 693 个氨基酸残基组成的蛋白质。推导的氨基酸序列与其他鳞翅目昆虫 PPO2 基因相应氨基酸序列有较高的同源性,该序列具有它们的 PPO 基因所共有的典型特征。组织特异性表达分析表明了该基因在野桑蚕 5 龄幼虫的血细胞、体壁、头部、精巢、卵巢、脂肪体和中肠等组织及其不同的发育阶段均有表达。这些结果为进一步研究野桑蚕酚氧化酶原基因的功能提供了分子基础。

关键词:野桑蚕;酚氧化酶原基因;克隆;序列分析;组织表达

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